ELSEVIER

Insect Biochemistry and Molecular Biology 34 (2004) 121–130

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Understanding and improving transgene stability and expression in insects for SIT and conditional lethal release programs

Alfred M. Handler *

Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, U.S. Department of Agriculture, 1700 S.W. 23rd Drive, Gainesville, FL 32608, USA

Received 2 December 2002; received in revised form 19 March 2003; accepted 7 August 2003

Abstract

Genetically transformed insect pests provide significant opportunities to create strains for improved sterile insect technique and new strategies based on conditional lethality. A major concern for programs that rely on the release of transgenic insects is the stability of the transgene, and maintenance of consistent expression of genes of interest within the transgene. Transgene instability would influence the integrity of the transformant strain upon which the effectiveness of the biological control program depends. Loss or intra-genomic transgene movement would result in strain attributes important to the program being lost or diminished, and the mass-release of such insects could significantly exacerbate the insect pest problem. Instability resulting in intra-genomic movement may also be a prelude to inter-genomic transgene movement between species resulting in ecological risks. This is less of a concern for short-term releases, where transgenic insects are not expected to survive in the environment beyond two or three generations. Transgene movement may occur, however, into infectious agents during mass-rearing, and the potential for movement after release is a possibility for programs using many millions of insects. The primary methods of addressing potential transgene instability relate to an understanding of the vector system used for gene transfer, the potential for its mobilization by the same or a related vector system, and methods required to identify transformants and determine if unexpected transgene movement has occurred. Methods also exist for preventing transposon-mediated mobilization, by deleting or rearranging vector sequences required for transposition using recombination systems. Stability of transgene expression is also a critical concern, especially in terms of potential epigenetic interactions with host genomes resulting in gene silencing that have been observed in plants and fungi, and it must be determined if this or related phenomena can occur in insects.

Keywords: Transgenic insects; Sterile insect technique; Transgene stability; Transposable elements; Epigenetics

1. Introduction

The ability to genetically transform insect pests presents a wide array of possibilities to create strains with specific attributes that can greatly improve existing biological control methods, as well as the development of novel new strategies for control (see Handler, 2002a). The effective use of such strains, however, relies on consistent and reliable expression of the integrated genes of interest, as well as maintenance of strain fitness and viability, especially under mass-rearing protocols. It is also essential that the transgene vector remain stably integrated, not only to maintain strain integrity, but also to prevent possible inter-species movement of the transgene into unintended hosts. Thus, the major areas of concern for the release of transgenic insects for sterile insect technique (SIT) and the release of conditional lethal (CLR) strains relate to risks associated with stability of the transgene vector and the expression of genes of interest within the vector. The potential for unintended transgene movement and its impact on program effectiveness and ecological risks must be considered in the context of several factors. These include the host insect, the vector system used for transformation, the methods and facilities for mass-rearing, and the anticipated persistence of the transformant strain in the field. The mechanisms and interactions that might result in strain instability will be a function of

* Tel.: +1-352-374-5793; fax: +1-352-374-5794.
E-mail address: handler@nersp.nerdc.ufl.edu (A.M. Handler).

0965-1748/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2003.08.005

the number of generations the strain is maintained under mass-rearing, and the generations it is expected to be in the field. For example, for programs such as SIT, the presence of the transgenic strain in the field is not expected to exceed the lifetime of the released insect (short-term single generation). While for CLR programs, such as release of insects with a dominant lethal (RIDL) (Thomas et al., 2000), autocidal biological control (ABC) (Fryxell and Miller, 1994), and similar systems (Heinrich and Scott, 2000; Horn and Wimmer, 2003) that rely on inherited lethality or sterility to suppress the released insect and its offspring, several generations will be required for the transgenic insects to be eliminated (short-term multi-generational).

Transgene instability would first be evident by intra-genomic movement of the vector, that could influence the integrity of the transformant strain upon which the effectiveness of the biological control program depends. If the transgene is lost or transposes to another site within the genome, strain attributes important to the program may be lost or diminished, and the mass-release of such insects could significantly worsen the targeted insect pest problem. Instability resulting in intra-genomic movement may also be a prelude to inter-genomic transgene movement between species resulting in ecological risks. While less of a concern for short-term releases, transgene movement into intermediary symbionts or infectious agents could occur during mass-rearing, allowing rapid movement into other hosts after release. For programs where many millions of insects are released, the potential for inter-species movement must be considered a statistical possibility.

The primary methods of addressing potential transgene instability relate to an understanding of the vector system used for gene transfer, the potential for its mobilization by the same or a related vector system, and methods required to identify transformants and determine if unexpected transgene movement has occurred. To prevent transposon-mediated mobilization, methods exist to delete or rearrange vector sequences critical for transposition post-integration using recombination systems such as FLP/FRT and Cre/loxP (see Rong and Golic, 2000). Another potential source of instability in transgene expression may result from epigenetic interactions with the host genome that have been well-documented as gene silencing systems in plants and fungi (see Martienssen and Colot, 2001). It is currently unknown if, and to what extent, gene silencing resulting from RNA-mediated methylation occurs in insect systems, and further understanding of this phenomenon will be essential.

2. Transposon-based vectors for gene transfer

An evaluation of risks associated with transgenic insect release and development of methods to abate these risks requires an understanding of the systems and processes used for gene transfer. The transformation systems available include those that result in the stable heritable integration of a transgene by germ-line transformation, while other systems include gene expression from transgenic symbionts (known as para-transgenesis), or by the extrachromosomal transient expression of a genetic system, usually mediated by a viral or bacterial system. For the applied use of gene expression systems in released insects, germ-line transformation typically mediated by a transposable element based system is currently the method of choice, and these are the systems we will focus on for this discussion.

A critical assessment of risks associated with strain stability requires a thorough knowledge of the behavior and regulatory properties of the vector used for genomic integration. The vectors currently used for non-drosophilid germ-line transformation are Class II transposable elements that are short terminal inverted repeat (TIR) transposons that transpose via a DNA-mediated intermediate in a cut-and-paste fashion (see Handler, 2001). These elements, generally, range in size from 1.3 to 3.2 kb and have a transcriptional unit within the TIR sequences that encodes a transposase molecule that acts at or near the termini to catalyze excision and transposition of the complete element. The ability of the transposase to act in *trans* has allowed the development of binary vector-helper systems where the mobile TIRs surround a marker gene and genes of interest within a vector plasmid, while the helper transposase, rendered immobile due to deletion of one or both of the terminal sequences, is provided on a separate plasmid (Rubin and Spradling, 1982). After introduction into germ cells, the helper mediates transposition of the vector into the genome, but since the helper is unable to integrate, it is lost after subsequent cell divisions allowing the vector to remain stably integrated. Thus, integrated vectors are typically defective non-autonomous transposons that require an exogenous source of transposase to be re-mobilized.

Short-term concerns for transgene stability generally relate to the potential for mobilization by the same or a related transposon system. The current transposons used for insect germ-line transformation, that include *piggyBac, Hermes, Minos*, and *mariner* (see Handler, 2001; Atkinson et al., 2001), all have closely related elements that exist in divergent species. For some, inter-species movement has very likely occurred by horizontal transmission, and the mechanisms involved in this movement provide a major area of concern for potential transgene movement.

2.1. *Hermes*

Hermes was discovered in *Musca domestica* and is a member of the *hobo, Activator, Tam3 (hAT)* family of transposons, and among functional transposons, is most closely related to *hobo* (Warren et al., 1994). Its existence was first postulated by the fact that *hobo* excision from plasmids occurred in the absence of *hobo* transposase in *Musca* (Atkinson et al., 1993), and the same finding was made for *hobo* in three tephritid fruit fly species (Handler and Gomez, 1996). *Hermes* has proven to be an effective transformation vector in *Drosophila* (O'Brochta et al., 1996), medfly (Michel et al., 2001), stablefly (O'Brochta et al., 2000) and several mosquito species. A unique aspect of *Hermes* transposition is that unusual recombinant integrations that include the entire vector plasmid were observed in the culicine mosquitoes, *Aedes aegypti* (Jasinskiene et al., 1998) and *Culex quinquefasciatus* (Allen et al., 2001), while only precise integrations were reported for an *Anopheles gambiae* cell line (Zhao and Eggleston, 1998). It has been postulated that some of these are replicative recombination events caused by interactions with related genomic *hAT* elements (Jasinskiene et al., 2000). A more direct functional relationship between these elements has been shown by plasmid and germline excision assays where *hobo* transposase mobilized both *Hermes* and *hobo* terminal sequence (Sundararajan et al., 1999). This is the first direct evidence for the cross-mobilization of related, yet distinct elements, and heightens the concern for *hAT* vector stability and transmission to non-target species.

2.2. *Minos* and *mariner*

Minos and *mariner* are members of the wide ranging *mariner/Tc* family. *Minos* is an effective vector system and has been tested in two non-drosophilids, the Mediterranean fruit fly (Loukeris et al., 1995) and *Anopheles stephensi* (Catteruccia et al., 2000). It is most closely related to *Tc* elements originally discovered in nematodes (Franz and Savakis, 1991), although direct interaction with these elements has not been reported. Embryonic and cell line transposition assays indicate that it has a wide range of function including insects and vertebrates (Klinakis et al., 2000; Zagoraiou et al., 2001).

Mariner was discovered in *D. mauritiana* (Medhora et al., 1988), and related elements have been found in a wide range of insects and other organisms (Robertson and MacLeod, 1993), and horizontal transmission has been suggested for the *Himar* element which exists in two insect orders (Robertson and Lampe, 1995). After transforming several *Drosophila* species (see Hartl et al., 1997), the *mariner Mos*1 element was used to transform *A. aegypti* (Coates et al., 1998) and *M. domestica* (Yoshiyama et al., 2000) and, notably, it has also transformed chickens and zebrafish (Sherman et al., 1998; Fadool et al., 1998). Given the demonstrated cross-mobilization between *hAT* transposons, and the wide ranging presence and function of *mariner/Tc* elements (Avancini et al., 1996; Robertson and MacLeod, 1993), vector stability and the potential for horizontal transmission is a concern for these vectors.

2.3. *piggyBac*

The *piggyBac* transposon (originally IFP2) is part of a subclass of elements that insert exclusively in TTAA target sites and excise only in a precise fashion (Elick et al., 1995; Fraser et al., 1996), and it has been used to transform more than 12 insect species spanning four orders including the Diptera, Lepidopteran, Coleoptera, and most recently, the Hymenopotera (see Handler, 2002b). Transformed species that may be controlled by SIT or CLR programs include medfly (Handler et al., 1998), caribfly (Handler and Harrell, 2001a), Oriental fruit fly (Handler and McCombs, 2000), pink bollworm (Peloquin et al., 2000), sheep blowfly (Heinrich et al., 2002), and *Anopheles albimanus* (Perera et al., in press), among several others. *piggyBac* was originally discovered as the causative agent of few polyhedra (FP) mutations in baculoviruses passed through the *Trichoplusia ni* TN-368 cell line (Fraser et al., 1983; Cary et al., 1989). Although it exists as a repetitive element in several *T. ni* cell lines (Fraser et al., 1996), it was not found in any other insect until recently when multiple complete, but non-functional *piggyBac* elements were discovered in the Oriental fruit fly, *Bactrocera dorsalis* (Handler and McCombs, 2000). *piggyBac* has since been discovered in eight sibling species of Bactrocera and three Spodoptera species (Handler, 2002b; Zimowska and Handler, unpublished). Thus, the existence of *piggyBac* in phylogenetically and geographically distinct species suggests that it has recently traversed insect orders by horizontal transmission, and probably exists in other species as well. The ability of *piggyBac* to transpose into an infectious baculovirus raises the possibility that its inter-species movement has been facilitated by a viral vector.

3. Identification and characterization of germ-line transgenics

Risk assessment evaluation of transgenic insects will depend initially on their identification and molecular characterization by methods that are efficient, reliable, and consistent so that changes in transgene integration site or expression can be definitively assessed. It is also important to efficiently distinguish transformant insects from non-transformed siblings so that potential

intra-genomic movement of the transgene can be distinguished from inter-genomic movement within the same species. It should also be possible to determine whether the transgene has been introduced into unrelated organisms, potentially transmitted by a non-vertical means of inheritance. If necessary, otherwise identical vectors may be marked with unique molecular tags (short DNA sequence) so their origin can be rapidly defined.

Transformant selection and identification in non-drosophilid insects originally used the eye color mutant-rescue selection routinely used in *Drosophila* (see Sarkar and Collins, 2000). Since mutant hosts and cloned wild type alleles are not widely available for other insects, use of fluorescent protein markers, which are dominant-acting visible neomorphs, have found common use in non-drosophilids (see Horn et al., 2002). The primary marker used thus far has been enhanced GFP (EGFP) under *polyubiquitin*, 3×P3, or *actin* promoter regulation, but other GFP variants and the DsRed fluorescent protein are finding use as well (Matz et al., 1999; Handler and Harrell, 2001b). These markers have the advantage of being easily and unambiguously identified, and they are less susceptible to position effects than white eye markers (Chalfie et al., 1994; Higgs and Lewis, 2000; Handler and Harrell, 1999). False positives and mis-identification are only problematic when indistinguishable autofluoresence occurs, and this can be minimized by using promoters limiting expression to specific tissues. An important benefit of using fluorescent protein markers is that they can be used as to detect released insects caught in traps (Handler, 2002a), and when regulated by broadly active promoters, they may be used to determine inter-species movement of a transgene.

The number and general integrity of vector integrations requires Southern DNA hybridization using restriction digests and probes that independently identify the 5′ and 3′ vector arms. Integration number should be consistent for both arms, with additional restriction digests used to clarify overlapping bands or incomplete digestions. Unusual insertions (e.g. recombination events) would typically result in anomalous hybridization patterns, though such patterns can also result from incomplete restriction enzyme digestion or from a mixed population of transformants having a varying number of unlinked transgene alleles. The use of Southern analysis as a test for transgene stability depends on unambiguous and consistent patterns of hybridization.

Insertion site sequencing allows the most definitive determination of transposon-mediated genomic integration. This is achieved most simply by inverse PCR methods that isolate the junction sites independently, or together as part of a single PCR product (Ochman et al., 1993). The method used depends on restriction sites available and whether the genome contains a single or multiple integrations (determined by Southern analysis). Proximal genomic sequence then allows a definitive determination of transposition by performing PCR on transformed and non-transformed host species genomes. The vector should be detected in the transformed genome, with only the empty insertion site in the non-transformed genome. These primers then become essential to rapidly defining integration stability in subsequent generations.

Chromosomal in situ hybridization also provides a definitive determination of a chromosomal integration and also allows a rapid determination of the number of genomic integrations and mapping. Hybridization to polytene chromosomes is most informative in species having a polytene map, but this is possible in only some dipteran species. An alternative is hybridization to mitotic chromosomes by fluorescent in situ hybridization (FISH).

4. Potential for intra- and inter-genomic movement

The primary concerns in evaluating transgenic insects for risk assessment and program effectiveness relate to transgene stability, which has implications for the integrity of the strain being released or studied, and potential risks if the transgene is transmitted to a non-host organism. For SIT and CLR programs, the primary risks for transgene instability relate to intra-genome movement, though the substantial number of organisms used in release programs raises the statistical possibility that inter-genomic movement may occur between species despite limited environmental exposure. For both transgene loss and transgene movement, a mobilizing system must exist within the transgenic host that is the same or similar to the transposon vector.

Transgene loss would eliminate the beneficial characteristics of the transgenic strain, and would be most devastating during mass-rearing if not rapidly detected before release. Intra-genome movement may result in differing position or dosage effects, or other genomic influences that can change the expressivity of the marker and/or the gene of interest. This could affect defined characteristics of the strain that may be essential to its effective use, and changes in marker gene expression may diminish the ability to detect transgenic insects for risk assessment and program analysis. Intra-genomic movement may also result in new integrations into lethal or semi-lethal sites that would diminish strain fitness or viability.

Inter-genome movement implies the horizontal transmission of a transgene between species, or different strains of the same species. The existence of nearly identical transposons in insects from different orders (e.g. *Himar* and *piggyBac*) has suggested that this is not

an uncommon natural phenomenon (Robertson and Lampe, 1995; Handler and McCombs, 2000). Such movement requires that the transposon is functionally autonomous, or that a cross-mobilizing system exists in the host genome that can de-stabilize the transgene, and the presence of a mechanism or vector that allows transmission of the transgene into a new genome. As discussed, the mobilizing system may be the same transposon as the gene transfer vector, or a related element. A primary question for risk assessment is how to detect and quantitate the activity of such a mobilizing system, and how to determine if it resides in the host genome or a co-habiting or infectious endosymbiont.

4.1. Evaluating the potential for transgene instability

Transgene instability would most directly result from the existence of the same transposon as the vector in a host species, and these could be straightforwardly detected by hybridization or PCR analysis. Identification of related cross-mobilizing systems can be more difficult since transposons, such as *hobo* and *Hermes*, may be functionally related but share insufficient structural similarity to make direct comparisons (Warren et al., 1994; Sundararajan et al., 1999). Thus, identification of such systems would rely on functional assays that can detect cross-mobilization. The most sensitive of these are embryonic or cell line excision assays performed in the absence of transposase, such as the *hobo* assays that implicated the existence of *Hermes* in *M. domestica* (Atkinson et al., 1993). Transposition assays could be similarly useful, but are more likely to detect only identical or nearly identical systems that can catalyze both excision and insertion (O'Brochta et al., 1994; Sarkar et al., 1997). A benefit of transposition assays is that if a nearly identical transposon is detected in a host strain, they can allow a rapid determination of function. For example, the functional *piggyBac* element was discovered in a *T. ni* cell line (Fraser et al., 1983), yet, none of the *piggyBac* sequences isolated by PCR from *T. ni* larval tissue are identical to the original element (Zimowska and Handler, unpublished). Negative data from transposition assays in *T. ni* embryos indicate that these in vivo elements are not functional (Lobo et al., 1999).

An important consideration for use of these assays is that the lack of a mobilizing system would be inferred by negative data; the absence of excision or transposition. Therefore statistical tests are necessary to conclude with confidence that a mobilizing system does not exist, or that mobilization is limited to the extent that it is not a concern. Controls for these tests should include mobility assays in strains of the host species that have a genomic source of transposase, such as a jumpstarter strain.

If transgene mobilization within the host species is possible, then an area of concern is the potential for its integration into a symbiotic, infectious, or predatory system that could mediate transmission to another host. These systems include procaryotic bacteria or viruses, or eucaryotic predators such as mites or wasps, that could also harbor a mobilizing system. The possibility for an infectious virus transmitting a eucaryotic transposon became evident by the discovery of *piggyBac* when it transposed into an infecting AcNPV genome (Fraser et al., 1983). Similar transpositions have been observed repeatedly from insect cell lines into several viral systems, as well as from a larval host (Jehle et al., 1998). While the *piggyBac* that transposed into AcNPV was an autonomous functional element, other TTAA elements discovered after transposition, such as *tagalong*, were defective elements that had to be cross-mobilized (Fraser et al., 1983). It is now important to determine whether transposon movement can occur from infectious agents or other potential vectors into a eucaryotic host genome, which would be required for horizontal transmission. For short-term release of transgenic strains, the highest likelihood would be for transposition into infectious agents during mass-rearing, that could have immediate consequences for inter-species movement into predatory organisms after release. Direct transposon movement from transgenic insects into predatory species or infectious agents after transgenic release would be less likely. Thus, a comprehensive assessment of potential vector mobilization into symbiotic or infectious agents during mass-rearing over numerous generations should be a high priority.

5. Mechanisms for *piggyBac* vector immobilization

5.1. Vector stability and the need for immobilizable vectors

Determining the presence and function of a transposable element in a wide range of insects and organisms they associate with will provide important information for assessing vector stability and the potential for breakdown of transgenic strains, as well as the potential for inter-genomic movement. Effectively addressing this potential was a primary criticism of the Environmental Assessment for the first experimental release of a transgenic insect (the pink bollworm, *Pectinophora gossypiella*) when public comment was solicited by USDA-APHIS-PPQ. Since it is not feasible to determine if and to what extent all potential hosts and all environmental situations will support transposon mobilization, experimental studies can only determine the potential for inter-species movement, and the relative risks to specific organisms if that were to occur. Eliminating the potential for transposon-mediated transposition may depend on the creation of vectors

that are immobilized after their initial integration. It is not inconceivable that the field release of transgenic insects will depend on use of immobilized vectors, though further studies into transposon behavior may eventually find that the risks for re-mobilization of non-autonomous transgenes in the field are negligible. However, these conclusions will not be made in the near-term, and they will certainly be relative to the effect of the genes being vectored, the host organism, and the area of release. For example, there will be minimal ecological concern for the release of transgenic insects that are sterile for SIT, with slightly more concern for CLR insects that are expected to die along with all of their offspring. For most transgenic releases in the foreseeable future, use of an immobilized vector, and data to support immobilization will present the best opportunity for these programs to go forward.

5.2. Vector immobilization

Transposon vector immobilization will be most simply achieved by the deletion or rearrangement of vector DNA required for mobility. This typically includes the inverted terminal repeats and associated subterminal DNA. For the *P* element, the minimal DNA requirements for mobility include 138 bp from the 5′ terminus and 216 bp from the 3′ terminus (Mullins et al., 1989). The minimal DNA requirements for mobility of most other transposon vectors are unclear, but at a minimum, the terminal TIRs are required in addition to subterminal inverted repeats if they exist. Typically adjacent DNA is required as well, but the actual sequences and length are specific to each transposon, and for most elements, the requisite terminal DNA for mobility must be empirically determined. A caveat is that these sequence requirements may differ for plasmid and chromosomal transposition, and thus vector modifications should be tested in both contexts.

Strategies to immobilize vectors include the internal deletion or rearrangement of subterminal sequences, or chromosomal inversions that displace one of the termini. These manipulations can be achieved by use of recombinase systems such as the *FRT*/FLP recombinase system from the 2μ plasmid of yeast (Andrews et al., 1985), though other systems such as the bacteriophage Cre-*loxP* system (Siegal and Hartl, 1996) may be similarly used. A functional *FRT* site consists of two 13 bp inverted repeats separated by an 8 bp spacer, which efficiently and specifically recombines with other sites in the presence of FLP recombinase. Importantly, specificity of *FRT* recombination depends on identical spacer sequences, so that *FRT* sites with different spacer sequences can be used in the same genome to confer predicted rearrangements (Senecoff and Cox, 1986). Depending upon their location, *FRT* recombination can occur within and between chromosomes causing translocations, inversions, insertions, duplications, and deletions (Golic and Lindquist, 1989; Golic et al., 1997; Golic and Golic, 1996). *FRT* recombination can also occur between plasmid DNA and chromosomes allowing insertion of the plasmid into a specific chromosomal target site. While there are advantages to using *FRT* target sites instead of transposon insertions for transformation, especially in terms of minimizing variable position effects, plasmid integration by *FRT* recombination has yet to be demonstrated.

5.3. FRT vectors for immobilization

The deletion or rearrangement of sequences required for movement can be achieved by surrounding these sequences with *FRT* sites as direct or inverted repeats within the vector, respectively. Recombination between the *FRT* sites would be catalyzed by FLP recombinase introduced by either injection of an FLP helper plasmid, FLP mRNA, or crosses to strains having a recombinase gene integration. Recombination between *FRT* direct repeats results only in deletion of sequences within the sites, with the rest of the vector and external marker DNA left intact. A secondary marker may be included within the *FRT* sites to visibly assess the deletion, though frequencies may be high enough so that PCR can be used to screen lines. Recombination between *FRT* inverted repeat sites results in inversions that "displace" or rearrange the critical sequences. This can be achieved within the vector, but can also occur between two independent vector integrations having the *FRT*s in opposite orientation resulting in a chromosomal inversion. There are relative advantages and disadvantages to these approaches in terms of practical implementation in non-drosophilid species.

5.3.1. Internal vector recombination

For internal vector deletions or rearrangements, one *FRT* site is required within the terminal sequence critical for movement, with another site positioned internally. Recombination can be monitored by having the sites surrounding a visible marker, such as *white*, that would be deleted by direct repeats, and this could be monitored as a somatic or germ-line event. For rearrangements mediated by inverted *FRT* sites, the internal *FRT* could be placed between the promoter and coding sequence, or within an intron, of a marker gene so that an internal inversion would disrupt its gene expression. A caveat for this strategy is the possibility that insertion of an *FRT* site into terminal sequences could negatively effect vector mobility, preventing the initial transformation event, and this may require testing various *FRT* insertion sites.

5.3.2. Chromosomal inversions

The second approach for vector immobilization relies on chromosomal inversions between two vector integrations. Single *FRT* sequences would be inserted into two vectors using the same transposon system, but in opposite orientation, or in two different vector transposons (e.g. *piggyBac* and *Hermes*) in either orientation. Host insects would be transformed with the vector pairs and selected for linked integrations on the same chromosome having the *FRT* sites in an inverted or opposite orientation. Use of the same transposon system would require the vectors having the same 5′–3′ (head–tail) orientation resulting in the *FRT* sites being in opposite orientation. Recombination would result in a conversion of the 5′ terminus of one vector for the 3′ terminus of the other, yielding head–head and tail–tail vectors, thus immobilizing both vectors. Use of different vector transposons would be simpler since sequential transformations will be more reliable (if same vector is used, the second transformation may destabilize the first integration). For different transposons, vector orientation would be irrelevant since conversion of either the 3′ or 5′ termini would immobilize both vectors, though *FRT* sites would still have to be in opposite orientation for a chromosomal inversion to occur. An added benefit of this scheme is that the chromosomal inversion will inhibit normal recombination, so if genes of interest are within the inversion, the strain will be further stabilized.

The advantage to this approach is that the *FRT* sites should not interfere with vector integration, and if properly positioned, the desired inversion and vector immobilization is highly feasible. The disadvantage to this approach is selecting transformants with linked integrations in the proper orientation. Increasing distance between *FRT* sites also negatively affects recombination frequency, with distances greater than 30 Mb in *Drosophila* resulting in negligible recombination (a single chromosome arm in *Drosophila* is ~30 Mb) (see Rong and Golic, 2000). Determining linkage by segregation of differently marked vectors should not be difficult, but determining orientation is more problematic for non-drosophilids. In *Drosophila*, and additional species that are being sequenced, a determination of vector orientation is possible by sequencing the insertion sites and determining chromosomal positions using the genome sequence database (that will also reaffirm linkage). Determining orientation in other insect species is presently not possible, and all lines having linked vectors would have to be tested with recombinase. Identifying successful inversions, however, can be simplified by having the *FRT* site, in at least one of the vectors, within a secondary marker as described for internal inversions. If placed in a non-translated region (e.g. transcript leader sequence or intron), the *FRT* inversion should eliminate the marker phenotype.

Another possibility is to use *FRT*s with a palindromic spacer, that would allow recombination in both vector orientations resulting in deletions, as well as inversions. In this strategy, both vector orientations could be used, but chromosomal deletions resulting from direct repeats would probably be lethal, allowing a rapid selection for the desired inversions.

5.3.3. FLP recombinase

A reliable method of providing recombinase is by crossing the *FRT* strain to another strain having one or more *hsp*70-recombinase integrations which is done routinely in *Drosophila* (see Rong and Golic, 2000). Having heat shock regulated recombinase allows some control over the level of recombination and, indeed, since *FRT* recombination can proceed in both directions, the major caveat to this mechanism is the re-insertion of the excised product which will occur if recombinase activity is not down-regulated. *Drosophila* experiments have also used injection of FLP mRNA, produced in vitro from a T7 promoter, which has the advantage of catalyzing high levels of germ-line recombination, without the need for additional inter-strain matings (Rong and Golic, 2000).

For either vector deletions or inversions, the expected recombination event would be verified in transformants by PCR to determine vector sequence. Vector immobilization then would be tested by crossing the lines to an appropriate jumpstarter strain having an integrated source of transposase and testing marker stability compared to controls. Testing vector stability as a function of marker phenotype can be assessed in somatic tissue or in the germ-line, though use of the white eye color marker is preferable for somatic events.

6. Epigenetic host genome–vector interactions

Unanticipated mobilization of a transgene will certainly affect strain stability, but another important consideration is epigenetic interactions between the transgene vector and the host genome that can influence transformant strain stability in terms of its fitness and expression of the desired phenotype. The first of these genomic interactions to be considered, that were discussed previously, relate to chromosomal position effects such as transcriptional interference and promoter suppression. These can be controlled or prevented to some extent by the use of transcription terminators and insulator elements, respectively, placed at appropriate sites within the vector (Bell et al., 2001). It is also likely that the effect of these interactions would be evident early on, with affected transgenic strains being selected against before further use in SIT or CLR programs.

Other potential interactions involve transcriptional gene silencing (TGS) and post-transcriptional gene suppression (PTGS), and these are of concern since their affects may not be apparent in early generations, or may occur discontinuously in a transgenic population (see Fire, 1999). These phenomena have been generally attributed to defense mechanisms against invasive nucleic acids in plants, typically from viruses, with double-stranded RNA (dsRNA) eliciting PTGS and TGS resulting from CpG methylation of promoter sequences (see Martienssen and Colot, 2001). Transposons are also major targets for methylation that can have a positive affect due to enhanced stability resulting from repressed transposase expression. For defective vectors, however, concern is focused on the silencing or diminished expression of genes within the vector. Gene silencing may also occur at particular insertion sites by a reverse enhancer effect resulting from proximal opposite-strand enhancers that promote dsRNA transcription. This could result in silencing of genes within the vector as well as homologous genes at chromosomal loci. Such epigenetic interactions could have a negative influence on marker gene expression that would impede identification of transformant organisms, and if it occurred in generations subsequent to primary transformant selection, monitoring of the release program and risk assessment analysis would be seriously affected. In a worse case scenario, there could be unintended use or release of ''unmarked'' transgenic organisms, especially if marker suppression occurred discontinuously in the population. Similarly, repression of genes of interest in transgenic populations could result in fertile males being used for SIT or loss of conditional lethality for CLR, and if undetected, could seriously exacerbate the pest problem after release.

Genomic interactions could also affect strain fitness and viability by mechanisms such as co-suppression or intercellular PTGS spreading, that result in repression of chromosomal genes vital to normal development, reproduction, or behavior (see Mlotshwa et al., 2002). Fitness can also be affected by transgene integrations that promote genomic rearrangements. Some *hobo* insertion sites are associated with chromosomal breakpoints in *D. melanogaster* (Lim, 1981), and a *mariner*-related element in humans is associated with a recombination hotspot resulting in duplications and deficiencies from unequal crossovers (Reiter et al., 1996).

The influence of CpG methylation on gene silencing in plants is well-documented, but it is very important to note that, presently, it is unknown if and to what extent this and associated mechanisms affect insects. Methylation occurs at very low levels in *Drosophila*, and it is generally associated with CpT and CpA dinucleotide sites during embryogenesis (see Lyko, 2001). Any influence by methylation or dsRNA on transgene silencing in *Drosophila* has not been documented, though RNA-mediated repression of the LINE-related I element activity has been suggested (Jensen et al., 1999). It is not unlikely, however, that methylation is more pronounced in some insects, and that transposons are differentially affected by PTGS, perhaps as a function of the genomic environment.

Suffice it to say, a high priority for research is the assessment of potential epigenetic effects on specific vectors in specific host species. Gene silencing or variable expression would eventually manifest itself during mass-rearing, and until such concerns are deemed inconsequential, careful monitoring of transgene presence and expression will be critical to program efficacy and safety. Similar to the way that the filter rearing system (Fisher and Caceres, 2000) controls for low level recombination in mass-reared genetic-sexing strains in medfly, a filter system may be used to maintain transgenic strains with consistent levels of gene expression.

7. Summary

Areas of concern for transgenic insect strains intended for mass-release in SIT and CLR programs primarily relate to the stability of the transgene vector and expression of marker genes and genes of interest within the vector. Both these attributes are essential to the maintenance of transgenic strain integrity, so that the desired phenotype is reliably and consistently expressed. Vector instability and/or repression of transgene expression would seriously compromise program effectiveness, possibly exacerbating the targeted problem, and potentially creating environmental risks by the unintended behavior or release of the transgenic strain. Potential vector instability mediated by mobilization or cross-mobilization by related transposon systems may be anticipated by surveys of the host species and associated organisms for the presence and function of such systems. A more reliable method of managing the potential for transposase-mediated movement is by creating new vectors that can be immobilized post-integration. These would rely on recombination systems that could delete or rearrange internal sequences necessary for transposition. A more daunting concern, however, relates to potential epigenetic interactions between transgene vectors and host genomes as observed in transgenic plants. While such interactions may have the positive benefit of repressing transgene movement, they also have the potential for repressing or silencing transgene markers and genes of interest so that identification and activity of the transgenic strain becomes unreliable. Use of insulator elements may prevent some types of repression, though continued research into transposon vector systems, potential host genomes, and their interactions will be essential for the effective use of transgenic insects for SIT and CLR.

Acknowledgements

Grateful appreciation is extended to Paul Eggleston and Grazyna Zimowska for comments on the manuscript, and support from the USDA-NRI Competitive Grants Program.

References

Allen, M.L., O'Brochta, D.A., Atkinson, P.W., Levesque, C.S., 2001. Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicinae). J. Med. Entomol. 38, 701–710.

Andrews, B.J., Proteau, G.A., Beatty, L.G., Sadowski, P.D., 1985. The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. Cell 40, 795–803.

Atkinson, P.W., Pinkerton, A.C., O'Brochta, D.A., 2001. Genetic transformation systems in insects. Annu. Rev. Entomol. 46, 317–346.

Atkinson, P.W., Warren, W.D., O'Brochta, D.A., 1993. The *hobo* transposable element of *Drosophila* can be cross-mobilized in houseflies and excises like the Ac element of maize. Proc. Natl. Acad. Sci. USA 90, 9693–9697.

Avancini, R.M., Walden, K.K., Robertson, H.M., 1996. The genomes of most animals have multiple members of the Tcl family of transposable elements. Genetica 98, 131–140.

Bell, A.C., West, A.G., Felsenfeld, G., 2001. Insulators and boundaries: versatile regulatory elements in the eukaryotic genome. Science 291, 447–450.

Cary, L.C., Goebel, M., Corsaro, H.H., Wang, H.H., Rosen, E., Fraser, M.J., 1989. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-Locus of nuclear polyhedrosis viruses. Virology 161, 8–17.

Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, C., Kafatos, F.C., Crisanti, A., 2000. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature 405, 959–962.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263, 802–805.

Coates, C.J., Jasinskiene, N., Miyashiro, L., James, A.A., 1998. *Mariner* transposition and transformation of the yellow fever mosquito *Aedes aegypti*. Proc. Natl. Acad. Sci. USA 95, 3742–3751.

Elick, T.A., Bauser, C.A., Principe, N.M., Fraser, M.J., 1995. PCR analysis of insertion site specificity, transcription, and structural uniformity of the Lepidopteran transposable element IFP2 in the TN-368 cell genome. Genetica 97, 127–139.

Fadool, J.M., Hartl, D.L., Dowling, J.E., 1998. Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish. Proc. Natl. Acad. Sci. USA 95, 5182–5186.

Fire, A., 1999. RNA-triggered gene silencing. Trends Genet. 15, 358–363.

Fisher, K., Caceres, C., 2000. A filter rearing system for mass-reared medfly. In: Tan Penang, K.H. (Ed.), Area-wide Control of Fruit Flies and Other Insects Pests. In: Joint Proceedings of the International Conference on Area-wide Control of Insect Pests and the Fifth International Symposium on Fruit Flies of Economic Importance. Malaysia, pp. 543–550.

Franz, G., Savakis, C., 1991. *Minos*, a new transposable element from *Drosophila hydei*, is a member of the Tc-1-like family of transposons. Nucleic Acids Res. 19, 6646.

Fraser, M.J., Smith, G.E., Summers, M.D., 1983. Acquisition of host-cell DNA-sequences by baculoviruses—relationship between host DNA insertions and FP mutants of *Autographa-californica* and *Galleria-mellonella* nuclear polyhedrosis viruses. J. Virol. 47, 287–300.

Fraser, M.J., Ciszczon, T., Elick, T., Bauser, C., 1996. Precise excision of TTAA-specific lepidopteran transposons *piggyBac* (IFP2) and *tagalong* (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. Insect Mol. Biol. 5, 141–151.

Fryxell, K.J., Miller, T.A., 1994. Autocidal biological control: a general strategy for insect control based on genetic transformation with a highly conserved gene. J. Econ. Entomol. 85, 1240–1245.

Golic, K.G., Lindquist, S.L., 1989. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. Cell 59, 499–509.

Golic, M.M., Rong, Y.S., Petersen, R.B., Lindquist, S.L., Golic, K.G., 1997. FLP-mediated DNA mobilization to specific target sites in *Drosophila* chromosomes. Nucleic Acids Res. 25, 3665–3671.

Golic, K.G., Golic, M.M., 1996. Engineering the *Drosophila* genome: chromosome rearrangements by design. Genetics 144, 1693–1711.

Handler, A.M., 2001. A current perspective on insect gene transfer. Insect Biochem. Mol. Biol. 31, 111–128.

Handler, A.M., 2002a. Prospects for using genetic transformation for improved SIT and new biocontrol methods. Genetica 116, 137–149.

Handler, A.M., 2002b. Use of the *piggyBac* transposon for germline transformation of insects. Insect Biochem. Mol. Biol. 32, 1211–1220.

Handler, A.M., Gomez, S.P., 1996. The *hobo* transposable element excises and has related elements in tephritid species. Genetics 143, 1339–1347.

Handler, A.M., Harrell, R.A., 1999. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Mol. Biol. 8, 449–458.

Handler, A.M., Harrell, R.A., 2001a. Transformation of the Caribbean fruit fly with a *piggyBac* transposon vector marked with polyubiquitin-regulated GFP. Insect Biochem. Mol. Biol. 31, 199–205.

Handler, A.M., Harrell, R.A., 2001b. Polyubiquitin-regulated DsRed marker for transgenic insects. Biotechniques 31, 820–828.

Handler, A.M., McCombs, S.D., 2000. The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. Insect Mol. Biol. 9, 605–612.

Handler, A.M., McCombs, S.D., Fraser, M.J., Saul, S.H., 1998. The lepidopteran transposon vector, *piggyBac*, mediates germline transformation in the Mediterranean fruitfly. Proc. Natl. Acad. Sci. USA 95, 7520–7525.

Hartl, D.L., Lohe, A.R., Lozovskaya, E.R., 1997. Modern thoughts on an ancyent marinere: function, evolution, regulation. Annu. Rev. Genet. 31, 337–358.

Heinrich, J.C., Scott, M.J., 2000. A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. Proc. Natl. Acad. Sci. USA 97, 8229–8232.

Heinrich, J.C., Li, X., Henry, R.A., Haack, N., Stringfellow, L., Heath, A., Scott, M.J., 2002. Germ-line transformation of the Australian sheep blowfly, *Lucilia cuprina*. Insect Mol. Biol. 11, 1–10.

Higgs, S., Lewis, D.L., 2000. Green fluorescent protein (GFP) as a marker for transgenic insects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, Florida, pp. 93–108.

Horn, C., Schmid, B.G.M., Pogoda, F.S., Wimmer, E.A., 2002. Fluorescent transformation markers for insect transgenesis. Insect Biochem. Mol. Biol. 32, 1221–1235.

Horn, C., Wimmer, E.A., 2003. A transgene-based, embryo-specific lethality system for insect pest management. Nat. Biotechnol. 21, 64–70.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S., James, A.A., Collins, F.H., 1998. Stable, transposon mediated transformation of the yellow fever mosquito, *Aedes aegypti*,

using the *Hermes* element from the housefly. Proc. Natl. Acad. Sci. USA 95, 3743–3747.

Jasinskiene, N., Coates, C.J., James, A.A., 2000. Structure of *Hermes* integrations in the germline of the yellow fever mosquito, *Aedes aegypti*. Insect Mol. Biol. 9, 11–18.

Jehle, J.A., Nickel, A., Vlak, J.M., Backhaus, H., 1998. Horizontal escape of the novel Tc1-like lepidopteran transposon TCp3.2 into Cydia pomonella granulovirus. J. Mol. Evol. 46, 215–224.

Jensen, S., Gassarna, M.P., Heidmann, T., 1999. Cosuppression of I transposon activity in *Drosophila* by I-containing sense and antisense transgenes. Genetics 153, 1767–1774.

Klinakis, A.G., Loukeris, T.G., Pavlopoulos, A., Savakis, C., 2000. Mobility assays confirm the broad host-range activity of the Minos transposable element and validate new transformation tools. Insect Mol. Biol. 9, 269–275.

Lim, J.K., 1981. Site-specific intrachromosomal rearrangements in *Drosophila melanogaster*: cytogenetic evidence for transposable elements. Cold Spring Harbor Symp. Quant. Biol. 45, 553–560.

Lobo, N., Li, X., Fraser, Jr.M.J., 1999. Transposition of the *piggyBac* element in embryos of *Drosophila melanogaster, Aedes aegypti* and *Trichoplusia ni*. Mol. Gen. Genet. 261, 803–810.

Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S., Savakis, C., 1995. Gene transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. Science 270, 2002–2005.

Lyko, F., 2001. DNA methylation learns to fly. Trends Genet. 17, 169–172.

Martienssen, R.A., Colot, V., 2001. DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science 293, 1070–1074.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., Lukyanov, S.A., 1999. Fluorescent proteins from nonbioluminescent *Anthozoa* species. Nat. Biotechnol. 17, 969–973.

Medhora, M.M., MacPeek, A.H., Hartl, D.L., 1988. Excision of the *Drosophila* transposable element *mariner*: identification and characterization of the *Mos* factor. EMBO J. 7, 2185–2189.

Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S., Gariou-Papalexiou, A., Zacharopoulou, A., O'Brochta, D.A., Atkinson, P.W., 2001. Hermes-mediated germ-line transformation of the Mediterranean fruit fly *Ceratitis capitata*. Insect Mol. Biol. 10, 155–162.

Mlotshwa, S., Voinnet, O., Mette, M.F., Matzke, M., Vaucheret, H., Ding, S.W., Pruss, G., Vance, V.B., 2002. RNA silencing and the mobile silencing signal. Plant Cell 14 (Suppl.), S289–S301.

Mullins, M.C., Rio, D.C., Rubin, G.M., 1989. *Cis*-acting DNA sequence requirements for P-element transposition. Cell 3, 729–738.

O'Brochta, D.A., Atkinson, P.W., Lehane, M.J., 2000. Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector. Insect Mol. Biol. 9, 531–538.

O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., 1994. Interplasmid transposition of *Drosophila hobo* elements in non-drosophilid insects. Mol. Gen. Genet. 244, 9–14.

O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., 1996. *Hermes*, a functional non-drosophilid insect gene vector. Genetics 142, 907–914.

Ochman, H., Ayala, F.J., Hartl, D.L., 1993. Use of polymerase chain reaction to amplify segments outside boundaries of known sequences. Methods Enzymol. 218, 309–321.

Peloquin, J.J., Thibault, S.T., Staten, R., Miller, T.A., 2000. Germline transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. Insect Mol. Biol. 9, 323–333.

Perera, O.P., Harrell, R.A., Handler, A.M., 2002. Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac/EGFP* transposon vector is routine and highly efficient. Insect Mol. Biol. 11, 291–297.

Reiter, L.T., Murakami, T., Koeuth, T., Pentao, L., Muzny, D.M., Gibbs, R.A., Lupski, J.R., 1996. A recombination hotspot responsible for two inherited peripheral neuropathies is located near a *mariner* transposon-like element. Nat. Genet. 12, 288–297.

Robertson, H.M., Lampe, D.J., 1995. Recent horizontal transfer of a *mariner* transposable element among and between Diptera and Neuroptera. Mol. Biol. Evol. 12, 850–862.

Robertson, H.M., MacLeod, E.G., 1993. Five major subfamilies of *mariner* transposable elements in insects, including the Mediterranean fruit fly, and related arthropods. Insect Mol. Biol. 2, 125–139.

Rong, Y., Golic, K., 2000. Site-specific recombination for the genetic manipulation of transgenic insects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, Florida, pp. 53–75.

Rubin, G.M., Spradling, A.C., 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218, 348–353.

Sarkar, A., Collins, F.H., 2000. Eye color genes for selection of transgenic insects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, Florida, pp. 93–108.

Sarkar, A., Coates, C.J., Whyard, S., Willhoeft, U., Atkinson, P.W., O'Brochta, D.A., 1997. The *Hermes* element from *Musca domestica* can transpose in four families of cyclorrhaphan flies. Genetica 99, 15–29.

Senecoff, J.F., Cox, M.M., 1986. Directionality in FLP protein-promoted site-specific recombination is mediated by DNA–DNA pairing. J. Biol. Chem. 261, 7380–7386.

Sherman, A., Dawson, A., Mather, C., Gilhooley, H., Li, Y., Mitchell, R., Finnegan, D., Sang, H., 1998. Transposition of the *Drosophila* element *mariner* into the chicken germ line. Nat. Biotechnol. 16, 1050–1053.

Siegal, M.L., Hartl, D.L., 1996. Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*. Genetics 144, 715–726.

Sundararajan, P., Atkinson, P.W., O'Brochta, D.A., 1999. Transposable element interactions in insects: crossmobilization of *hobo* and *Hermes*. Insect Mol. Biol. 8, 359–368.

Thomas, D.T., Donnelly, C.A., Wood, R.J., Alphey, L.S., 2000. Insect population control using a dominant, repressible, lethal genetic system. Science 287, 2474–2476.

Warren, W.D., Atkinson, P.W., O'Brochta, D.A., 1994. The *Hermes* transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the *hobo, Ac*, and *Tam*3 (*h*AT) element family. Genet. Res. Camb. 64, 87–97.

Yoshiyama, M., Honda, H., Kimura, K., 2000. Successful transformation of the housefly, *Musca domestica* (Diptera: Muscidae) with the transposable element, *mariner*. Appl. Entomol. Zool. 35, 321–325.

Zagoraiou, L., Drabek, D., Alexaki, S., Guy, J.A., Klinakis, A.J., Langeveld, A., Skavdis, G., Mamalaki, C., Grosveld, F., Savakis, C., 2001. In vivo transposition of *Minos*, a *Drosophila* mobile element, in mammalian tissues. Proc. Natl. Acad. Sci. USA 98, 11474–11478.

Zhao, Y.G., Eggleston, P., 1998. Transformation of an *Anopheles gambiae* cell line mediated by the *Hermes* mobile genetic element. Insect Biochem. Mol. Biol. 28, 213–219.